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Intracapsular Feeding by Embryos of the Gastropod Genus *Littorina*

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Abstract. Many gastropod species develop within egg capsules within which larvae are provided with extraembryonic nutrients. Species with encapsulated development frequently have transitory embryonic organs, such as "larval kidneys," that may represent specializations for consumption of intracapsular nutrition. Larvae of *Littorina* species with nonplanktonic, encapsulated development consume intracapsular albumen, but they lack obvious morphological modifications for albumen consumption. To determine the mechanism and location of protein uptake, larvae of seven species of *Littorina* (*L. keenae, L. littorea, L. plena, L. saxatilis, L. scutulata, L. sitkana, L. subrotundata*) were exposed to solutions of either fluorescently labeled protein (FITC-bovine serum albumen) or ferritin. Under fluorescence microscopy, larvae of all species with encapsulated, nonplanktonic development displayed strong regional affinity for FITC in the ciliated cells of the velum, whereas hatched larvae of planktotrophic *Littorina* species did not. Transmission electron microscopy of epithelial cells of nonplanktotrophic veligers exposed to ferritin supported the interpretation that localized affinity for labeled protein indicated endocytotic protein uptake. Planktotrophic *Littorina* and *Littorina* with encapsulated, nonplanktonic development were shown to share equivalent velar width/larval length ratios during early embryonic development, whereas a literature search suggested that in other nonplanktotrophic prosobranchs the velum is relatively smaller than in planktotrophs. Retention of a large velum in *Littorina* that develop entirely within egg capsules may facilitate feeding on intracapsular protein, in the absence of specialized assimilative organs found in other species with encapsulated development.

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Introduction

Marine invertebrates exhibit a remarkable variety of reproductive and developmental modes both within and among taxa, and this variation provides a powerful comparative means of studying the integration of development, life history, and evolution. One of the best-known dichotomies in invertebrate development is between species with larvae that must feed in the plankton to grow and attain metamorphic competence (*planktotrophic*), and species that reach competence without feeding in the plankton (*nonplanktotrophic*) (Thorson, 1946; Jablonski and Lutz, 1983; Strathmann, 1985). Another important distinction can be made between species with entirely planktonic development, and species that spend all or part of development in egg capsules (*planktonic, encapsulated nonplanktonic*, and *mixed* development, respectively) (Thorson, 1946; Pechenik, 1979; Perron, 1981).

Larvae of many species with encapsulated, nonplanktonic development are morphologically similar to larvae of related planktotrophic species, and retain structures that presumably had an ancestral role in larval swimming and feeding. For example, larvae of most gastropod molluscs with nonplanktonic, encapsulated development possess a velum (*e.g.*, Fretter and Graham, 1962; Buckland-Nicks *et al.*, 1973; Strathmann, 1978; Hadfield and Iaea, 1989), which is the primary larval structure that planktotrophic molluscan larvae use in swimming and food collection (Strathmann and Leise, 1979). The velum may be smaller in species that lack free-living larvae than in planktotrophic species (Jägersten, 1972; Webber, 1977; Rivest, 1983; but see Hadfield and Iaea, 1989), and it may have different patterns of ciliation (*e.g.*, Lyons and Spight, 1973; Hadfield and Iaea, 1989). These changes in gastropod larval morphology have been interpreted as the loss of complex, ancestral planktotrophic features due to relaxation of stabilizing selection, and as functional modifications that en-

hance performance during development in the egg capsule (Fretter and Graham, 1962; Lyons and Spight, 1973; Hadfield and Iaea, 1989).

Egg capsules protect offspring (Shuto, 1974; Spight, 1977; Pechenik, 1984; Hawkins and Hutchinson, 1988; Rawlings, 1990, 1996) and retain progeny within suitable adult habitat (Wells and Wells, 1962; Chapman, 1965; Pechenik, 1979); in many species, capsules also provide a nutrient-rich environment for developing embryos (Fretter and Graham, 1962; Fioroni, 1977, 1988). Larvae of many gastropods with mixed or entirely encapsulated development have transitory structures that may represent specializations for consumption of nutritive materials such as albumen or nurse eggs (Portmann, 1955; Portmann and Sandmeier, 1965; Cather and Tompa, 1972; Lyons and Spight, 1973; Rivest, 1983, 1992; Rivest and Strathmann, 1995). However, in many species the mechanisms of consumption of intracapsular nutrition are poorly known. Likewise, the extent to which planktotrophic larval characters have been modified in species with nonplanktonic, encapsulated larvae is not fully understood, and has not previously been addressed by comparing closely related taxa with contrasting developmental modes.

The gastropod genus *Littorina,* the periwinkle snails, contains ~19 species found in the high-shore zone throughout the northern Atlantic and Pacific oceans (Reid, 1989; Reid *et al.,* 1996). The genus contains both planktotrophic and nonplanktotrophic species (see Reid, 1989, for review); molecular phylogenetic evidence supports planktotrophy as the ancestral state within the genus (see Rumbak *et al.,* 1994). All species of *Littorina* undergo early development in egg capsules. Planktotrophic species have "mixed" development: early developmental stages are contained in complex pelagic capsules (Fig. 1A), within which each larva is encased in an individual egg envelope (Fig. 1A) from which it emerges just prior to hatching from the capsule as a swimming, planktotrophic veliger. Other species lack a planktonic stage altogether, metamorphosing from veligers to juveniles within benthic or brooded egg masses. Egg capsules of these nonplanktotrophic species are filled with granular albumen (Fig. 1B) that larvae consume during development (Buckland-Nicks *et al.,* 1973). *Littorina* veligers have been reported to lack albumen-absorbing larval kidneys (Rivest, 1981; 1992), and the site and mechanism of albumen consumption in encapsulated embryos and larvae of nonplanktotrophic *Littorina* species have not been established.

The objectives of this study were to (1) determine the location of nutrient assimilation by larvae of encapsulated, nonplanktonic species in the gastropod genus *Littorina*; (2) investigate the extent to which congeneric planktotrophs share similar patterns of assimilation; and (3) compare the larval functional feeding morphologies of planktotrophic *Littorina* and their congeners that develop entirely within egg capsules.

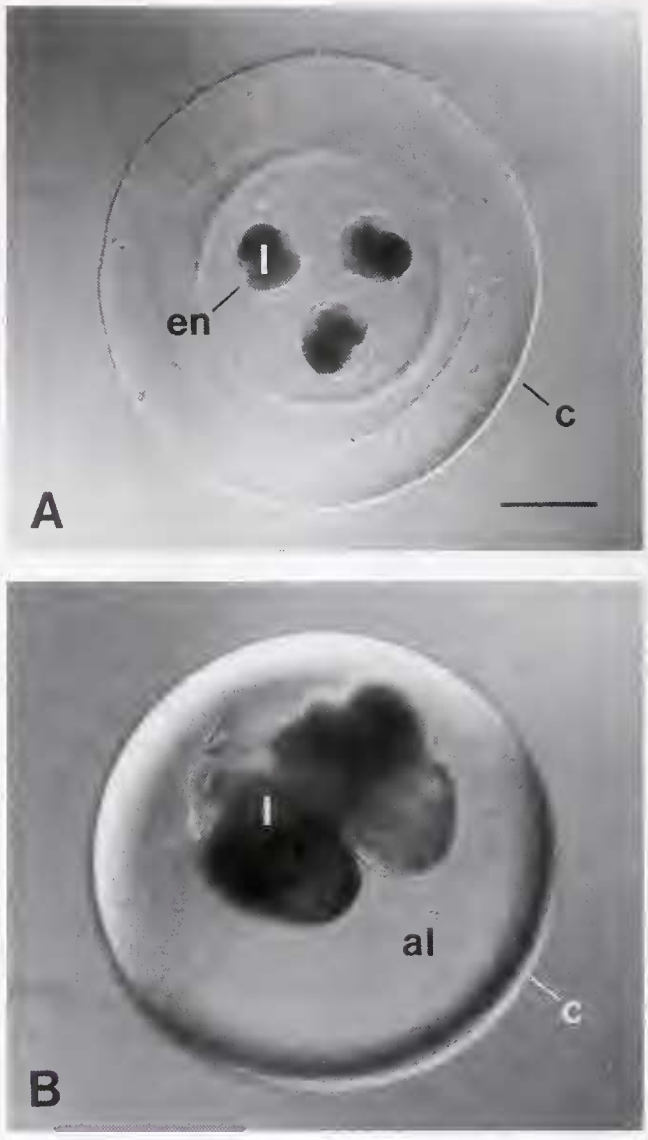

Figure 1. (A) Planktonic egg capsule of *Littorina scutulata,* a species with planktotrophic development, containing three prehatching veliger-stage larvae surrounded by individual egg envelopes. (B) Albumen-filled capsule of *Littorina saxatilis,* a species with encapsulated, nonplanktonic development. Capsule was removed from the oviduct of a gravid female. al, albumen; c, capsule; l, larva; en, egg envelope. Scale bar = 200 μm.

Materials and Methods

Terminology

Because the words *embryo* and *larva* can have multiple interpretations in taxa with complex life histories, I use the terminology for early developmental stages as defined by McEdward and Janies (1993). *Embryo* refers to developmental stages from fertilized egg to gastrula; *larva* describes

any premetamorphic stage with recognizable larval features (*e.g.*, the prototroch), regardless of whether stages are free-living or not.

Spawning and larval rearing

Seven *Littorina* species were used in these experiments: four planktotrophs (*L. littorea* (Linnaeus, 1758), *L. keenae* Rosewater, 1978, *L. plena* Gould 1849, *L. scutulata* Gould, 1849) and three species with encapsulated, nonplanktonic development (*L. saxatilis* (Olivi, 1792), *L. sitkana* Philippi, 1846, *L. subrotundata* (Carpenter, 1864)) (Table I). Egg capsules of planktotrophic species and species with benthic egg masses (*L. sitkana, L. subrotundata*) were obtained by placing live, freshly collected adult animals into mesh-walled containers (<1 mm diameter mesh size), and immersing the containers in vigorously aerated seawater for 1 to 7 days. Egg capsules of the nonplanktotrophic species *L. saxatilis,* which broods larvae to metamorphosis in the oviduct, were obtained by cracking adult animals with needle-nose pliers and removing capsules from the brood chamber.

Larvae of planktotrophic species were reared at concentrations of about one larva per milliliter in 0.45 μm filtered seawater changed every 4 days. Planktotrophic larvae were fed *ad libitum* on a mixture of the single-celled algae *Isochrysis galbani* (CCMP #1324 [T-ISO]) and *Dunaliella tertiolecta* (CCMP #1320 [DUN]). Egg masses of species with encapsulated, nonplanktonic development were maintained in glass dishes of filtered (0.45 μm) seawater at 12°C.

Fluorescence microscopy

To test for regions of affinity for labeled albumen protein, embryos and larvae of three species with encapsulated, nonplanktonic development (*L. saxatilis, L. sitkana, L. subrotundata*) were removed from their capsules at developmental stages from early cleavage to metamorphosis. Fine forceps were used to free embryos and larvae from capsules. Veliger larvae of four planktotrophic species (*L. littorea, L. planaxis, L. plena, L. scutulata*) were examined both before and after they hatched from the egg capsule. Because the egg envelope that surrounded earlier developmental stages of planktotrophs could not be removed without damage to developing larvae, early larval stages of one planktotrophic species (*L. plena*) were exposed to test solutions while they were still in the egg envelope.

Embryos and larvae were placed in solutions of bovine serum albumen labeled with fluoroscein isothiocyanate (FITC-BSA, Sigma #A-9771), a solution useful for demonstrating receptor-mediated endocytosis of proteins (Rivest, 1992; Rivest and Strathmann, 1995). FITC-BSA was also made in the laboratory from commercially available BSA and FITC (Sigma #F-7250) using the methods of Rivest (1981). To remove unconjugated FITC, FITC-BSA was dialyzed for 24 h against several changes of filtered (0.45 μm) seawater or treated with excess charcoal for 30 min (Rivest, 1981).

Embryos and larvae were placed in test solutions of 10–1000 μg/ml FITC-BSA in filtered seawater at 12°C for 15 min to 24 h, then rinsed in filtered seawater for periods ranging from 1 to 48 h. Controls were exposed to test solutions containing filtered seawater only, unlabeled BSA, or unconjugated FITC. Experimental and control embryos and larvae were examined with an Olympus epifluorescence microscope fitted with an FITC filter set (Omega Optics stock number XF23, excitation maximum 485 nm, emission 535 nm).

Transmission electron microscopy

To identify regions active in protein uptake, veligers of one species with encapsulated, nonplanktonic development

Table I

Species, development and collection information for Littorina *utilized in this study*

Species, Authority	Mode[1]	Collection locality	Collection habitat
Littorina littorea (Linnaeus, 1758)	P	Woods Hole, MA	Rocky shoreline
		Mystic, CT	
Littorina keenae Rosewater, 1978	P	Monterey, CA	Rocky shoreline
Littorina plena Gould, 1849	P	Charleston, OR	Rocky shoreline
Littorina scutulata Gould, 1849	P	Charleston, OR	Protected estuary
		Monterey, CA	Rocky shoreline
Littorina saxatilis (Olivi, 1792)	NP	Woods Hole, MA	Rocky shoreline
		Mystic, CT	
Littorina sitkana Philippi, 1846	NP	Charleston, OR	Estuarine marsh
		Friday Harbor, WA	Rocky shoreline
Littorina subrotundata (Carpenter, 1864)	NP	Charleston, OR	Estuarine marsh

[1] P = planktotrophic, NP = nonplanktotrophic.

(*L. sitkana*) were removed from their capsules and prepared for transmission electron microscopy, using the methods of Rivest and Strathmann (1995) with minor modifications. Larvae were placed for 10 min in a solution of 0.05% osmium tetroxide and 3% glutaraldehyde in 0.1 *M* (pH 7.35) phosphate buffer, with the osmolarity raised to 990 mOsM with sucrose. Next, larvae were placed in a solution of 3% glutaraldehyde in 0.1 *M* phosphate buffer with the osmolarity raised to 990 mOsM with sucrose for 1 h, after which an equal volume of 10% EDTA was added (to dissolve the shell) and larvae were fixed for another hour. Larvae were postfixed for 1 h at room temperature in 2% osmium tetroxide in 1.25% sodium bicarbonate, then dehydrated in an ethanol series. Finally, specimens were exchanged in propylene oxide, embedded in epoxy resin, and thin sections were cut on a Reichert Ultracut E ultramicrotome. Sections were picked up on Butvar films on 200-μm hex grids, stained with uranyl acetate and lead citrate (after Reynolds, 1963), and examined with a Philips CM 12 electron microscope.

To determine whether protein was assimilated into velar cells, some larvae were exposed to a solution of 1 mg/ml ferritin (Sigma catalog #F4503) in filtered seawater for 12 h and rinsed in seawater for 2 h prior to fixation. Ferritin is an electron-dense protein useful as a marker for endocytosis (Rivest, 1981). Five ferritin-exposed larvae and four controls were examined with transmission electron microscopy.

Measurement of velar aspect ratios

Freshly laid egg capsules of *L. plena* (planktotrophic) were transferred to filtered seawater and reared at room temperature. When larvae had reached the veliger stage (judged by the appearance of the velum), one larva was haphazardly chosen from each capsule. Brooded capsules of one species with encapsulated, nonplanktonic development (*L. saxatilis*) were removed from the adult, and several veligers from each brood were haphazardly chosen for measurements. Velar width and total length were measured on each larva, and dimensions of these measurements are shown in Figure 2. Larvae were traced with a camera lucida attached to a compound microscope, and measurements were made using a SummaSketch II digitizing pad and the software package SigmaScan for Windows (Jandel Corporation). To control for developmental stage, only early veligers of *L. saxatilis* (lacking well-developed tentacles or propodia, similar in degree of development to prehatching *L. plena*) were measured.

To compare the relative size of the velum and to compensate for size differences between larvae of the two species, the "velar aspect ratio" (VAR) was calculated by dividing velar width by total length of each larva. VARs were calculated for 64 *L. plena* and 60 *L. saxatilis* veligers. To compare VARs of other planktotrophic and nonplanktotrophic prosobranch gastropods, the literature was searched to find drawings or photographs of prosobranch veligers from many taxa. Images were chosen based on two criteria, developmental stage and orientation. Because comparisons of *Littorina* with different developmental modes were made when both species were fully developed, but early, veligers (see above), I selected images of planktotrophic species if the larvae were described as prehatching or newly hatched, and I rejected images of nonplanktotrophic species if the larvae were described as mature or near hatching. If multiple stages were pictured, I used the earliest veliger stage. Images were used only if both the full width of the velum and the length of the larva were depicted. However, because orientation varied considerably among images, measurements of length were necessarily somewhat subjective.

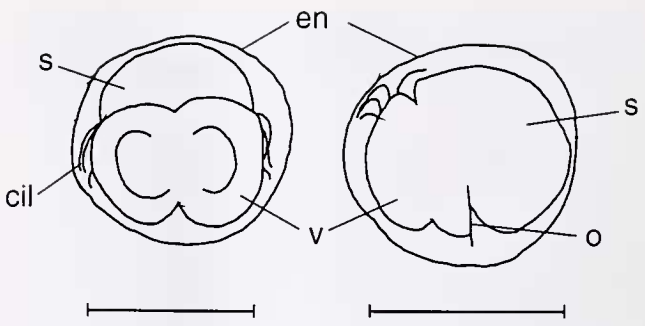

Figure 2. Camera lucida drawings of a prehatching larva of *Littorina plena* indicating measured dimensions: velar width (left on figure; apical view of larva) and larval length (right; lateral view). en, egg envelope; cil, cilia of velum; o, operculum; s, shell; v, velum). Larval length bar (on right) = 110 μm.

The velar aspect ratio of each image was measured with a SummaSketch II digitizing pad and SigmaScan for Windows software as described above for *Littorina*. If an image included a scale bar, it was used to calibrate measurements; otherwise, the aspect ratio was measured without units.

Ideally, statistical analyses would take into account the degrees of phylogenetic relatedness among all taxa; failure to do so may artificially inflate the sample size and hence the degrees of freedom (Martins and Hansen, 1996). Although there has been much recent interest in higher gastropod phylogeny (*e.g.*, Bieler, 1992; Ponder and Lindberg, 1996; Ponder and Lindberg, 1997), there is little consensus regarding relationships at or above the family level (Harasewych *et al.*, 1997; Ponder and Lindberg, 1997). Therefore, for the purposes of this study, species were not considered individually but were sorted by family. VARs were averaged within families if measurements were made on more than one species in a family, and average VARs were graphed for comparison of planktotrophs and nonplanktotrophs. Six families in this data set were represented by both planktotrophic and nonplanktotrophic species, presumably representing six independent losses of planktotrophy.

To compare velar sizes among developmental modes, mean VARs of planktotrophs and nonplanktotrophs of these six families were compared by using a paired Student's *t* test.

Measurement of cilia length

To determine whether ciliary length and growth differed between species with contrasting developmental modes, freshly spawned egg capsules of three planktotrophs (*L. keenae, L. plena, L. scutulata*) and egg masses of one species with encapsulated, nonplanktonic development (*L. sitkana*) were collected, transferred to filtered seawater, and reared at 12°C. Planktotrophs were observed from first cleavage through hatching; they were initially examined every 2–3 h and at intervals of about 1 d or longer in later development. The nonplanktotrophs, which developed more slowly, were observed from first cleavage through reaching a fully formed veliger; they were initially examined daily and during later development at intervals of several days. Larvae were immobilized under a coverslip and examined on a compound microscope equipped with a camera lucida. The cilia were drawn at their greatest extensions and the images were then digitized (as described for velar aspect ratios) to measure greatest length. In addition, veligers of *L. saxatilis* were removed from their capsules and placed in seawater to determine whether larvae could swim.

Results

Fluorescence microscopy

Encapsulated, nonplanktonic developers. None of the experimental eggs or embryos exhibited FITC fluorescence after exposure to FITC-BSA, prior to the trochophore stage. In all species with encapsulated, nonplanktonic development (*Littorina saxatilis, L. sitkana, L. subrotundata*), FITC-BSA fluorescence was first seen at the trochophore stage, when it was confined to the ciliated cells of the prototrochal band (prototroch; Fig. 3A, B). At the late trochophore stage (before the appearance of eyespots, foot, or the larval shell) and the veliger stage (with shell, foot, eyespots), FITC fluorescence was confined to the ciliated cells of the developing velum (Fig. 4A, B), which are derived from the ciliated cells of the prototroch (Fretter and Graham, 1962). Fluorescence was not evident in other cells of the pretrochal region or in the developing structures of the head (tentacles, eyespots; Fig. 5A–D). Fluorescence was seen in the ciliated cells of the velum of larvae exposed to FITC-BSA throughout development, until the velum was resorbed prior to hatching. FITC fluorescence in these cells was concentrated in small (<2 μm) vacuoles visible inside cells (Fig. 6).

In addition to the ciliated cells of the velum, three other larval regions showed FITC-BSA fluorescence. In older larvae with a well-developed foot, the ciliated cells of the rejection band of the foot fluoresced in a manner qualitatively similar to prototrochal/velar fluorescence. Small, isolated points of fluorescence, similar to the vacuoles seen in prototrochal/velar cells, were occasionally seen in the mantle and other parts of the foot as well. In mature veligers of all nonplanktotrophs, two small areas of FITC fluorescence appeared just posterior to the ciliated band of the velum on either side of the head (Fig. 7; "larval kidneys"). These areas autofluoresced in control larvae, but the intensity of autofluorescence was low and varied considerably among larvae. In mature, fully formed veligers and in posthatching juveniles, FITC fluorescence appeared in the gut as well (Fig. 8A, B). Newly hatched juveniles did not exhibit FITC fluorescence except in the gut.

Control embryos and larvae did not fluoresce when exposed to filtered seawater only or to BSA in filtered seawater, except for autofluorescence in the operculum and larval kidneys. Larvae exposed to unconjugated FITC displayed FITC fluorescence in multiple regions including the foot, shell gland, viscera, and velum, probably due to adherence of FITC to cell proteins. FITC fluorescence faded in most areas when larvae were rinsed in filtered seawater for >24 h. Unconjugated FITC also adhered to the shell and operculum of mature larvae.

Planktotrophs. Among larvae of four planktotrophic species exposed to test solutions containing FITC-BSA at the stage of emerging from the egg envelope (but prior to hatching from the egg capsule), none showed fluorescence in the velum (Fig. 9A, B). The planktotrophic *L. plena* did not exhibit FITC-BSA fluorescence at any stage when larvae in their egg envelopes were soaked overnight in FITC-BSA solutions, suggesting that either (1) the egg envelope is not permeable to large proteins (molecular weight of BSA is about 88,000 kDa), or (2) if the envelope is permeable to proteins, early stages do not exhibit affinity at a visually detectable level. Egg capsules of planktotrophic species contained a gel-like material outside the egg envelopes (Fig. 1A). However, this material did not appear to be protein-rich because no schleiren lines were seen when capsules were opened in seawater. Hatched veligers of all four planktotrophs exhibited fluorescence in the gut after exposure to FITC-BSA solutions (Fig. 9A, B). Control larvae exposed to filtered seawater only or to BSA in seawater showed autofluorescence in the operculum only.

Transmission electron microscopy

The ciliated cells of the velum of larvae exposed to ferritin contained numerous endosomes 1–5 μm in diameter filled with an electron-dense material that was by far the darkest material in unstained sections (Fig. 10A). This material was identified as ferritin because it consisted of densely packed particles of uniform shape about 7–8 nm in diameter (Fig. 10B), consistent with the size and appearance

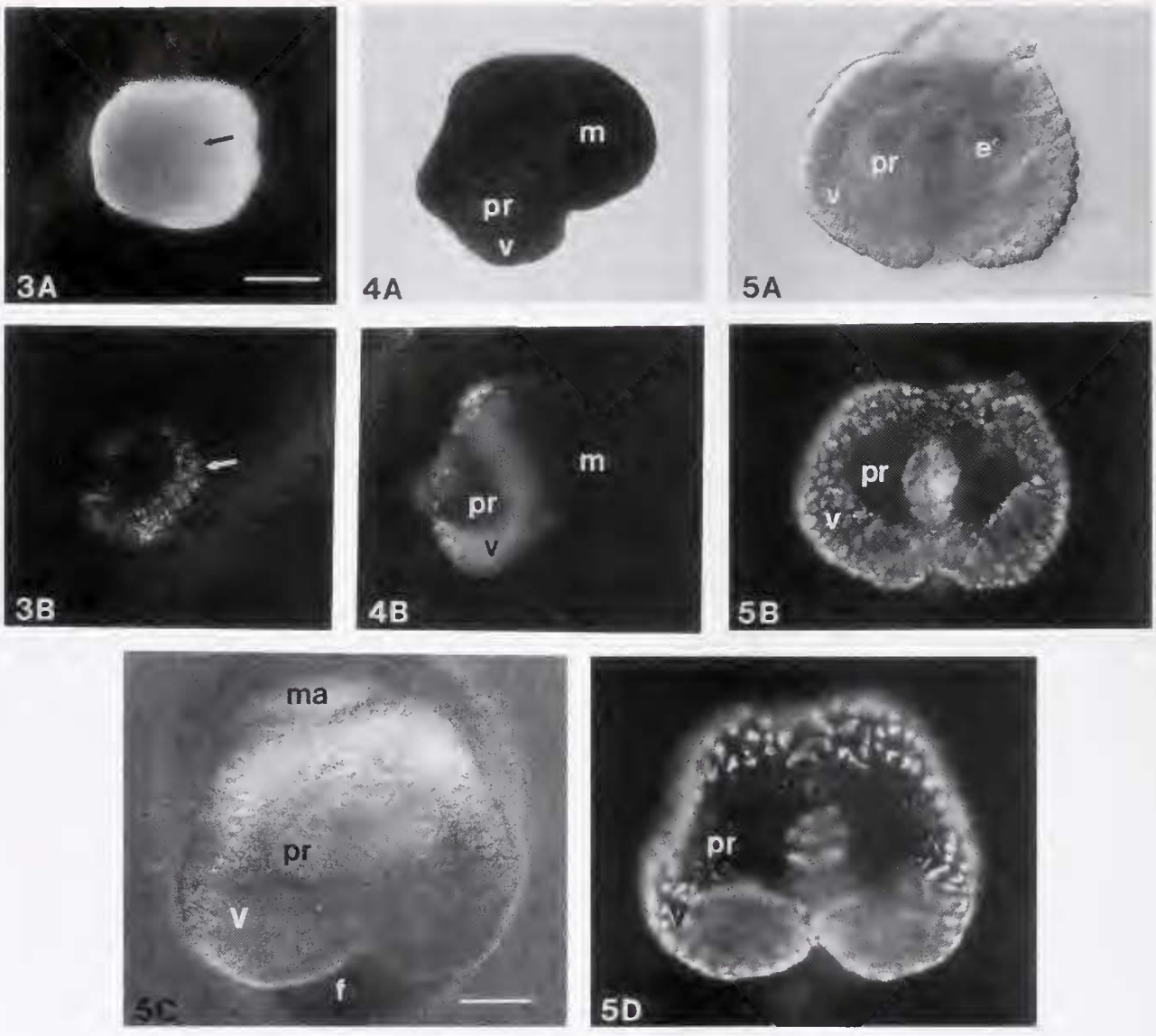

Figure 3. (A) Excapsulated trochophore larva of *Littorina saxatilis*, viewed obliquely from the apical end under transmitted light (dark field). Larva was exposed to fluorescently labeled albumen for 12 h. Arrow indicates position of prototroch (cilia are not visible). (B) Same larva viewed with epifluorescence microscopy and FITC filter set. Only the cells of the prototroch (arrow) are fluorescent, indicating localized affinity for FITC-labeled albumen. Scale bar = 70 μm.

Figure 4. (A) Excapsulated early veliger of *Littorina saxatilis* viewed from the side under transmitted light (bright field). Larva has been exposed to fluorescently labeled albumen for 12 h. (B) Same larva viewed with epifluorescence microscopy and FITC filter set. Only the ciliated cells of the velum are fluorescent; no fluorescence appears in the pretrochal region or visceral mass. m, visceral mass; pr, pretrochal region; v, velum (v is positioned on ciliated band). Scale bar (on Fig. 3) = 70 μm.

Figure 5. (A) Velum of excapsulated, mature veliger of *Littorina saxatilis* (viscera and foot have been removed) viewed under transmitted light (bright field). Larva was exposed to fluorescently labeled albumen for 12 h. (B) Same partial larva, viewed with epifluorescence microscopy and an FITC filter set. The ciliated velar cells are brightly fluorescent, whereas the pretrochal region exhibits no fluorescence. The fluorescent area in the center of the velum is continuous with the ciliated band of velar cells, and may represent cells of the apical plate (see Raven, 1958, pp. 143–145) or an extension of the ciliated band. (C) Excapsulated, mature veliger of *Littorina sitkana*, viewed head-on under transmitted light and epi-illumination. Larva was exposed to fluorescently labeled albumen for 12 h. (D) Same larva, viewed with epifluorescence microscopy and FITC filter set. Ciliated velar cells are brightly fluorescent, and some fluorescence can be seen (out of focus) in the ciliated rejection band of the foot. No fluorescence is seen in the pretrochal region or mantle. e, eyespot; f, foot; ma, mantle; pr, pretrochal region; v, velum (v is positioned on ciliated band). Scale bars; A–B (on Fig. 3A) = 70 μm, C–D (on Fig. 5C) = 50 μm.

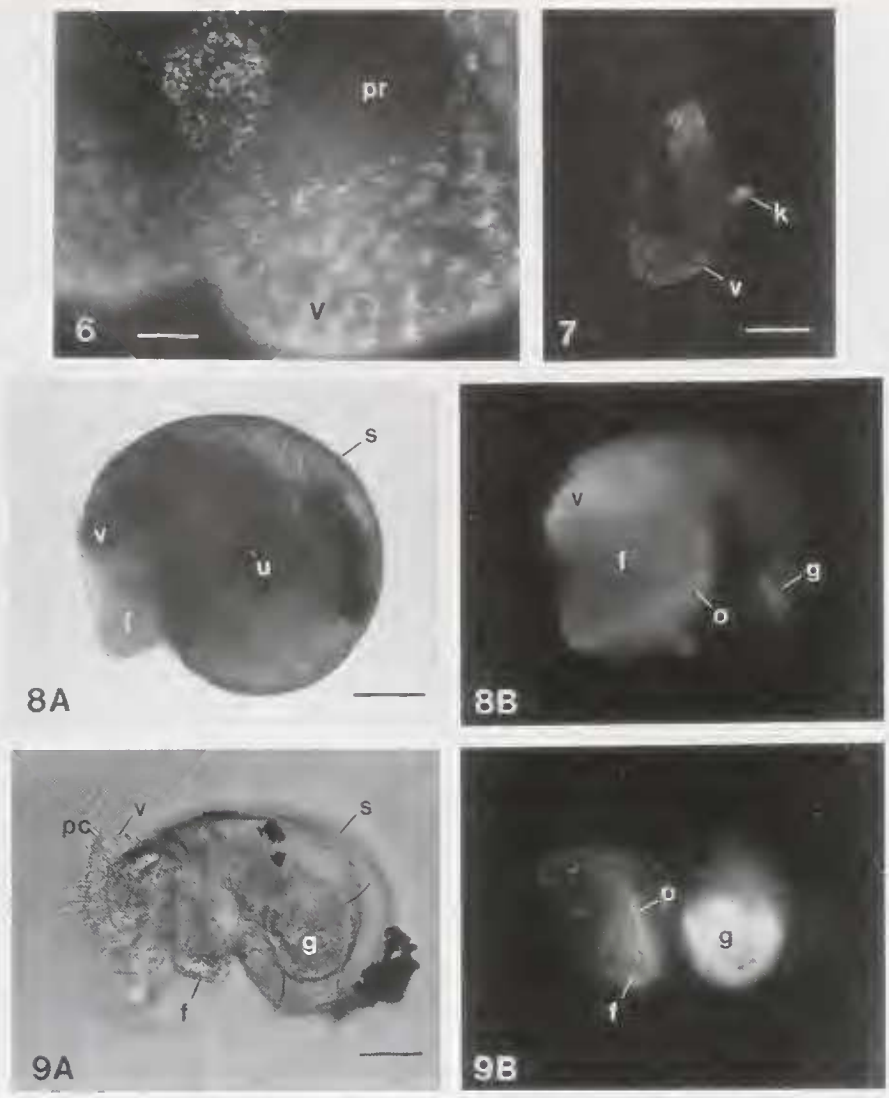

Figure 6. High-magnification view of velum of mature *Littorina saxatilis* larva viewed with epifluorescence microscopy (FITC filter set). Larva was exposed to fluorescently labeled albumen for 12 h. Fluorescence appears in the ciliated band of velar cells but not the pretrochal region. Fluorescence is concentrated in small (<2 μm) vacuoles within the prototrochal cells. pr, pretrochal region; v, velum (positioned on ciliated band). Scale bar = 20 μm.

Figure 7. Veliger of *Littorina saxatilis*, exposed to FITC-BSA and viewed laterally under fluorescent light with FITC filter set. The orientation of this larva is similar to Fig. 3. Immediately posterior to the ciliated band is one of two symmetrically positioned fluorescent regions (larval kidneys) that were found in fully formed veligers. k, larval kidney; v, velum. Scale bar = 75 μm.

Figure 8. (A) Mature veliger of *Littorina saxatilis*, close to hatching, viewed under transmitted light. Larva was exposed to fluorescently labeled albumen for 12 h. (B) Same larva viewed under fluorescent light with FITC filter set. Fluorescence can be seen in the ciliated cells of the velum, the autofluorescent operculum, and in a short region of the gut. The dim illumination of the foot is due to light reflected from the operculum. f, foot; g, gut; o, operculum; s, shell; u, umbilicus; v, velum. Scale bar = 150 μm.

Figure 9. (A) Newly hatched veliger of *Littorina plena* (planktotroph), viewed under transmitted light. Larva was exposed to fluorescently labeled albumen for 12 h. (B) Same larva viewed under fluorescent light with FITC filter set, showing strong FITC fluorescence in the gut. The operculum is autofluorescent, and the foot is dimly illuminated by light reflected from the operculum. Note absence of fluorescence in the velum. f, foot; g, gut; o, operculum; pc, prototrochal cilia; s, shell; v, velum. Scale bar = 25 μm.

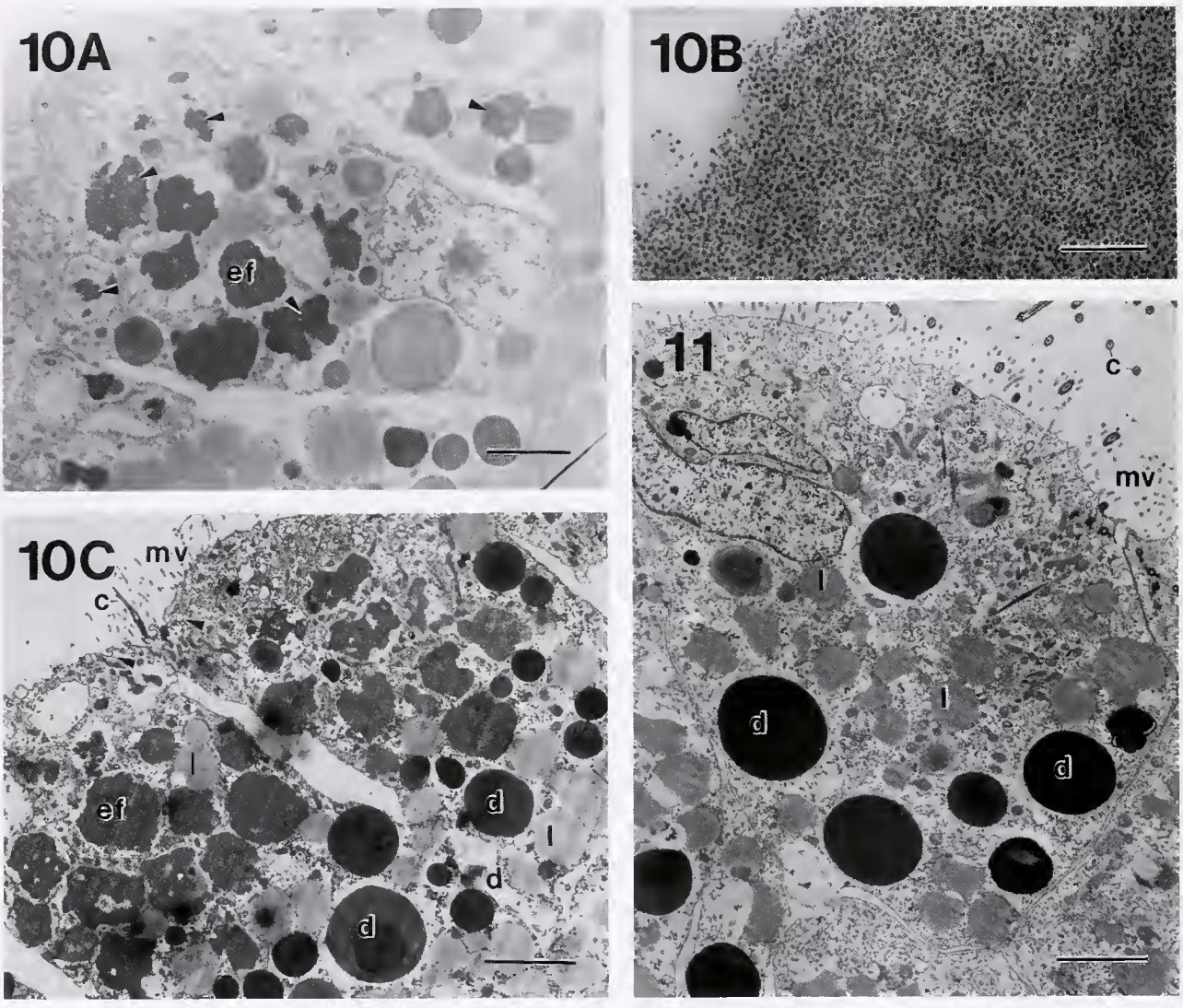

Figure 10. Transmission electron micrographs of the velum of *Littorina sitkana* exposed to ferritin for 12 h. (A) Unstained section of a ciliated cell of the velum. The plasma membrane is to the upper left. Cells contain numerous irregularly shaped endosomes 1–5 μm in diameter that are filled with a dark (electron-dense) granular material. One endosome is labeled, and other concentrations of ferritin are indicated with arrows. (B) High-magnification view of the electron-dense material contained within the endosomes in (A), identified as ferritin by its uniform shape and size (approximately 8 nm diameter, consistent with the size of ferritin molecules) and its absence from control larvae. (C) Stained section of a larva exposed to ferritin, with probable endocytotic vesicles (arrows), light body (tentatively identified as lipid), dark body (tentatively identified as yolk), and numerous endosomes (one is labeled) similar in size, shape, and appearance to endosomes appearing in the unstained section shown in (A). Endosomes in (C) contain the same granular material as those in (A). Note that in the stained section, endosomes and dark bodies are similar in electron density. Exterior of the larva is to the upper left. c, cilium; d, dark body; ef, endosome containing electron-dense material (ferritin); l, light body; mv, microvilli. Scale bars; A, 4 μm; B, 100 nm; C, 4 μm.

Figure 11. Transmission electron micrograph of ciliated cells of the velum of a *Littorina sitkana* larva removed from the egg capsule and soaked in filtered seawater for 12 h prior to fixation; larva NOT exposed to ferritin. Cilia, numerous microvilli, light bodies, and dark bodies are labeled. Note that dark bodies are the most electron-dense material in this section, and that ferritin-containing endosomes are lacking. Exterior of the larva is to the upper right. c, cilium; d, dark body; l, light body; mv, microvilli. Scale bar = 3 μm.

of the electron-dense core of ferritin molecules (Gider *et al.*, 1995). Ferritin-filled endosomes were also abundant in stained sections of ferritin-exposed larvae (Fig. 10C). Other distinctive features of stained sections of velar cells were numerous microvilli and invaginations at the cell surface (Fig. 10C), small (0.1–0.3 μm in diameter) vesicles imme-

diately inside the cell surface (Fig. 10C), "light bodies" tentatively identified as lipid, and "dark bodies" tentatively identified as yolk on the basis of the lack of a surrounding membrane (Fig. 10C).

Ciliated velar cells of larvae not exposed to ferritin contained many similar features, including surface cilia and microvilli and light and dark bodies (Fig. 11). Sections of control larvae were distinguished by the absence of ferritin-filled endosomes; ferritin or ferritin-like electron-dense material was not seen in velar sections of any of four control larvae examined.

At higher magnification, the cell membrane of ciliated velar cells of larvae exposed to ferritin appeared to be endocytotically active. Probable endocytotic profiles were evident in the plasma membrane (Fig. 12A), and ferritin-filled endosomes appeared immediately inside the cells (Fig. 12A, 12A inset). Nonciliated epithelial cells of ferritin-exposed larvae shared some features with ciliated cells of the velum and foot, including the presence of microvilli and light bodies. However, ferritin was not present in these cells (Fig. 12B) even when ciliated and nonciliated cells were immediately adjacent to each other, as in ciliated and nonciliated areas of the foot (Fig. 12C).

Velar aspect ratios

The velar aspect ratios (VARs) of early *Littorina saxatilis* (nonplanktotroph) veligers were not significantly different from the velar aspect ratio of prehatching *L. plena* (planktotroph) veligers (Student's two-sample t test, $P = 0.77$; Fig. 13A). The VARs of planktotrophs from the literature were generally greater than VARs of confamilial nonplanktotrophs, although there was considerable overlap (Table II; Fig. 13B). VARs of planktotrophs were significantly greater than nonplanktotrophs when average VARs were compared among planktotrophs and nonplanktotrophs from within six families (Fig. 13B; paired Student's t test, $P = 0.02$).

Cilia length

Velar cilia of planktotrophic *Littorina* larvae grew faster than cilia of *L. sitkana*, and reached a much greater total length (Fig. 14). Observations of cilia length in additional species with encapsulated, nonplanktonic development (*L. saxatilis, L. subrotundata*) were consistent with this pattern. The velum of these species bore cilia that appeared to be simple rather than compound, and when removed from their egg capsules, veligers of these species rotated on the bottom of the dish but did not swim.

Discussion

Two lines of evidence indicate that the ciliated cells of the velum and foot of *Littorina* with encapsulated, nonplanktonic development are active in localized uptake of capsular proteins. First, these cells fluoresced brightly after exposure to fluorescently labeled albumen, clearly indicating regional affinity for labeled protein. Affinity strongly suggested uptake, because fluorescently labeled vacuoles were interspersed with cell structures such as lipid droplets in optical sections. Second, ciliated cells of the velum and rejection band of the foot of larvae exposed to ferritin solutions contained large amounts of electron-dense material identifiable as ferritin by the size of molecules, whereas adjacent areas of the foot did not contain ferritin; likewise, no ferritin was seen in control larvae. The absence of ferritin from nonciliated cells of the foot (which did not fluoresce after exposure to FITC-BSA) further supports the hypothesis that regional FITC-BSA fluorescence indicates localized uptake of intracapsular albumen.

Other larval structures have been associated with the endocytotic uptake of capsular proteins by gastropods. These include the larval kidneys of many prosobranch species (Rivest, 1992), the "pedal cell complex" of neritoideans (Rivest and Strathmann, 1995), and the podocyst of some pulmonates (Cather and Tompa, 1972), all of which assimilate protein *via* receptor-mediated endocytosis (Cather and Tompa, 1972; Rivest, 1981, 1992; Rivest and Strathmann, 1995). Structures such as the pedal cell complex and larval kidneys are unique to encapsulated larval forms, and in species with mixed development these structures are resorbed before or soon after hatching (Rivest, 1992; Rivest and Strathmann, 1995). Thus, it has been proposed that such structures may have evolved to facilitate intracapsular development by enhancing capsular nutrient uptake (Rivest, 1992; Rivest and Strathmann, 1995) or respiration (Cather and Tompa, 1972; Rivest and Strathmann, 1995). The velum, in contrast, is an ancestral structure that is present in all *Littorina* and functions in swimming and particle capture in planktotrophic species; therefore, intracapsular protein uptake by the velum of nonplanktotrophic *Littorina* likely represents an exaptation of an ancestral character to the evolutionarily novel (within the genus) condition of developing to metamorphosis in the egg capsule.

In many taxa, egg capsules provide a nutrient-rich environment for developing embryos (Rivest and Strathmann, 1995), in addition to other functions such as protecting offspring from environmental stress and predation (Shuto, 1974; Spight, 1977; Pechenik, 1984; Hawkins and Hutchinson, 1988; Rawlings, 1990, 1996) and retaining progeny within suitable adult habitat (Wells and Wells, 1962; Chapman, 1965; Pechenik, 1979). Albumen is a common nutrient source among gastropods, and in many taxa albumen is rich in proteins, carbohydrates, or free amino acids (Hörstmann, 1956; De Mahieu *et al.*, 1974; Rivest, 1992; Stöckmann-Bosbach and Althoff, 1989; Penchaszadeh and Rincón, 1996). Albumen has been implicated in larval nutrition through a decline in nutrient content of the fluid during development, and by a correlated increase in larval lipid and

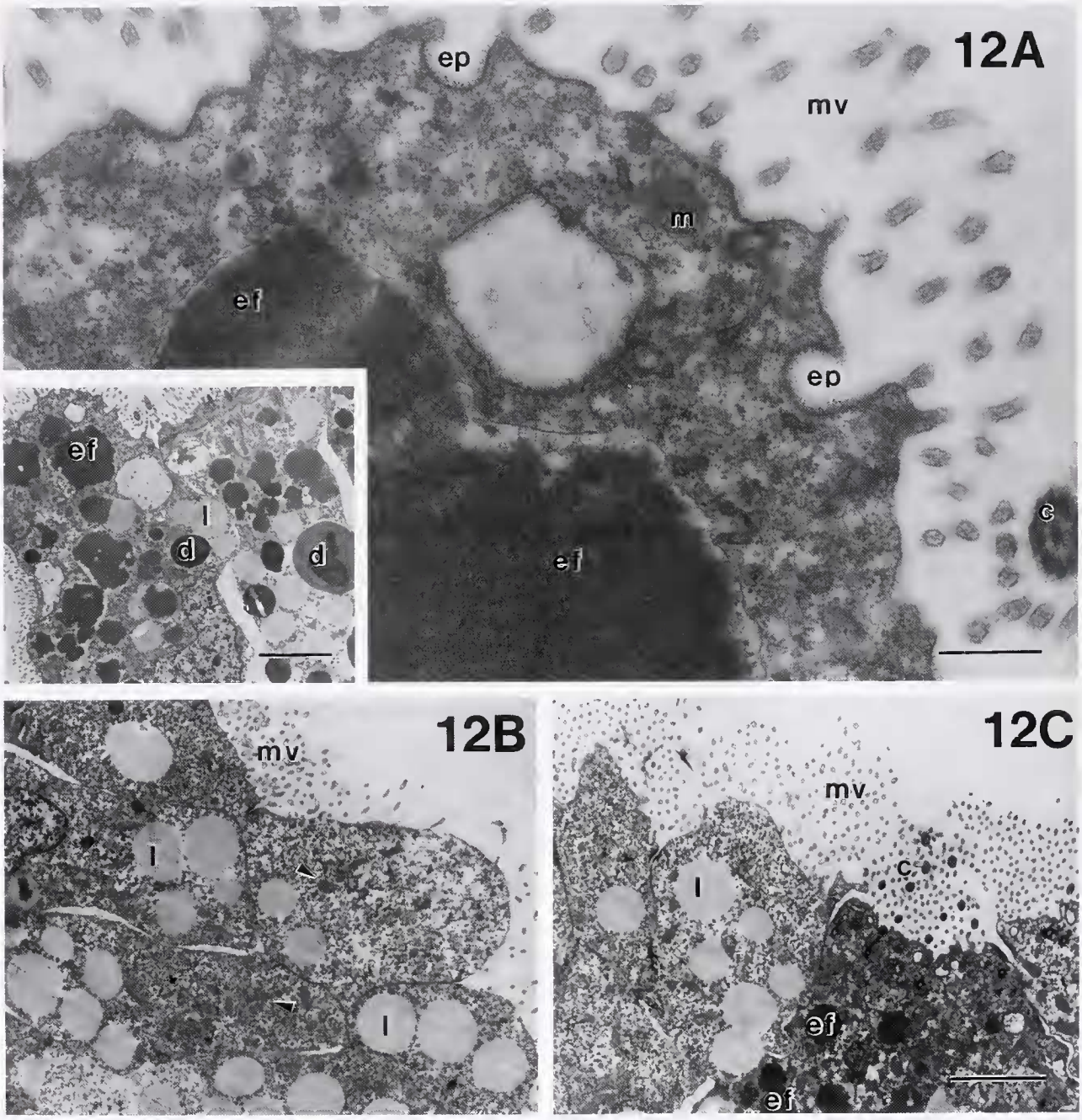

Figure 12. Transmission electron micrographs of sections from the velum and foot of *Littorina sitkana* larvae exposed to ferritin for 12 h. (A) High-magnification view of cell surface of a prototrochal cell of the velum, showing a prototrochal cilium, surface microvilli, a mitochondrion, and an endosome containing electron-dense material (ferritin). Two endocytotic profiles are evident at the cell surface membrane. Inset; lower magnification view of the same area of the larva. Magnified area shown in 12A is the upper left corner of the inset. (B) Cells from the foot of larva exposed to ferritin for 12 h. Cells contain light bodies and mitochondria (arrows). Numerous microvilli are visible on the cell surface, but cilia are not evident. Cells contain no ferritin. (C) Cells from the foot of a second larva also exposed to ferritin for 12 h. This image shows the junction of two cell types, from the ciliated rejection band of the foot (right) and adjacent, nonciliated foot cells (left). Ferritin-containing endosomes are visible in the ciliated cells but are not seen in adjacent, nonciliated cells. c, cilium; d, dark body; ef, endosome containing ferritin; ep, endocytotic profile; l, light body; mv, microvilli; m, mitochondrion. Scale bars; A, 0.5 μm; A inset, 5 μm; B and C, 3 μm.

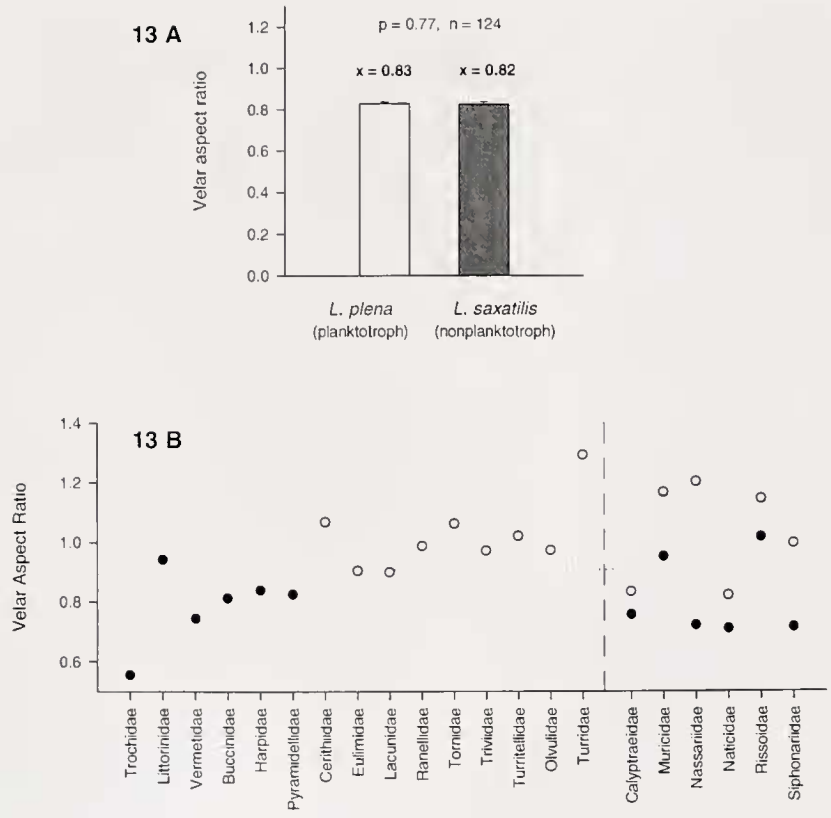

Figure 13. (A) Mean velar aspect ratios of 64 early veligers of *Littorina plena* (planktotroph, open bar) and 60 early *L. saxatilis* veligers (nonplanktotroph; shaded bar). Error bars are 95% confidence intervals. (B) Mean velar aspect ratios of planktotrophs (open circles) and nonplanktotrophs (closed circles) from 21 gastropod families. Error bars are not shown because most families are represented by single species (see Table I). The six families to the right of the vertical dotted line are represented by both planktotrophic and nonplanktotrophic species. The horizontal dotted line represents the mean velar aspect ratio of all families combined.

protein content (Hörstmann, 1956; Morrill, 1964; Raven, 1972; Morrill *et al.*, 1976; Taylor, 1983). Considerable increases in larval size are also correlated with disappearance of capsular albumen in many gastropod taxa (Rasmussen, 1951; Buckland-Nicks *et al.*, 1973; Clark *et al.*, 1979; Clark and Jensen, 1981; Losse and Greven, 1993). The nutritive importance of albumen varies in different taxa, depending on egg size, volume of albumen, nutritive content of albumen, and the presence or absence of additional food sources such as nurse eggs (Pechenik *et al.*, 1984; Miloslavich, 1996; Penchaszadeh and Rincón, 1996).

Intracapsular albumen is thought to be of nutritive importance to developing *Littorina* with encapsulated, nonplanktonic development for two reasons. First, albumen of one species, *L. saxatilis,* is dominated by a single protein of molecular weight 80,000 kDa that disappears from the intracapsular fluid during development (Losse and Greven, 1993). Second, larvae of these species increase in size during development; Buckland-Nicks *et al.* (1973) reported that *L. sitkana* increases from an egg size of 175 μm to a hatching length of 575 μm, presumably due to consumption of albumen. Velar albumen assimilation is likely to be important to larval nutrition, because the surface area of the velum is a large proportion of the larval surface area during early development (see Fig. 4). In addition, assimilation by the cells that will form the velum begins early in development, prior to the development of other structures implicated in assimilation (the foot, gut, and larval kidneys). Assimilation *via* the foot and gut may be of greater importance later in development, when the velum is being resorbed. Larval kidneys are unlikely to be as important in protein assimilation as other structures; their small size and dim fluorescence after exposure to FITC-BSA, which may account for previous reports of their absence in *Littorina sitkana* (Rivest, 1981, 1992), suggests that assimilation by the larval kidneys is negligible relative to velar assimilation.

The velum may take up other substances in addition to proteins. Losse and Greven (1993) noted considerable fluorescence in the gut, hepatopancreas, and velum of *L. saxatilis* veligers exposed to FITC-labeled dextrans, and the intracapsular fluid of some gastropods contains carbohydrates and free amino acids that may be nutritive (*e.g.*,

Table II

Taxa utilized in comparisons of velar aspect ratios among planktotrophs and nonplanktotrophs

Family	Genus/Species	Mode[1]	VAR[2]	References
Buccinidae	*Engoniophos unicinctus*	NP	1.205	Miloslavich and Penchaszadeh, 1994
	Neptunea antiqua	NP	0.418	Pearce and Thorson, 1967
Calyptraeidae	*Calyptraea chinensis*	NP	1.074	Lebour, 1936
	Calyptraea trochiformis	NP	0.779	Cañete and Ambler, 1992
	Crepidula aduncа	NP	0.47	Moritz, 1939
	Crepidula fornicata	P	0.834	Fretter and Graham, 1962
Cerithiidae	*Cerithiopsis tubercularis*	P	0.924	Lebour, 1993a
	Cerithiopsis barleei	P	1.135	Lebour, 1993a
	Triphora perversa	P	1.144	Lebour, 1993a
Eulimidae	*Eulima distorta*	P	0.829	Thorson, 1946
	Pelseneeria stylifera	P	0.982	Thorson, 1946
Harpidae	*Morum onuscus*	NP	0.839	Hughes, 1990
Lacunidae	*Lacuna vincta*	P	0.900	Lebour, 1937
Littorinidae	*Bembicium vittatum*	NP	0.943	Black *et al.*, 1994
Muricidae	*Chicoreous ramosus*	NP	1.599	Soliman, 1991
	Concholepas concholepas	P	1.169	DiSalvo, 1988
	Nucella lapilus	NP	0.817	Fretter and Graham, 1962
	Nucella lamellosa	NP	0.880	Lyons and Spight, 1973
	Nucella crassilabrum	NP	0.625	Gallardo, 1979
	Nucella canaliculata	NP	0.780	Lyons and Spight, 1973
	Nucella emarginata	NP	0.861	Lyons and Spight, 1973
	Trophon muricatus	NP	1.102	Lebour, 1936
Nassariidae	*Bullia digitalis*	NP	0.720	da Silva and Brown, 1985
	Nassarius incrassatus	P	1.150	Lebour, 1931a
	Nassarius reticulatus	P	1.257	Lebour, 1931a
Naticidae	*Natica catena*	NP	0.574	Thorson, 1946
	Natica (Lunatida) pallida	NP	0.843	Thorson, 1946
	Natica (Lunatida) nitida	P	0.822	Thorson, 1946
Olvulidae	*Simnia barbarensis*	P	0.973	Main, 1974
Pyramidellidae	*Boonea (Odostomia) impressa*	NP	0.825	White *et al.*, 1985
Ranellidae	*Cabestana spengleri*	P	0.987	Reidel, 1992
Rissoidae	*Cingula semicostata*	NP	1.018	Lebour, 1934
	Rissoa sarsii	P	1.134	Lebour, 1934
	Rissoa membranacea	P	1.000	Lebour, 1934
	Rissoa parva	P	1.247	Lebour, 1934
	Rissoa guerini	P	1.202	Lebour, 1934
Tornidae	*Tornus subcarinatus*	P	1.064	Lebour, 1936
Triviidae	*Trivia europa*	P	0.971	Lebour, 1931b
Trochidae	*Calliostoma granulatum*	NP	0.707	Ramón, 1990
	Margarites helicinus	NP	0.393	Holyoak, 1988
	Tegula funebratis	NP	0.566	Moran, 1997
Turritellidae	*Turritella communis*	P	1.022	Kennedy and Keegan, 1992
Turridae	*Haedropleura septangularis*	P	1.205	Lebour, 1936
	Philbertia (Comarmondia) gracilis	P	1.380	Lebour, 1993b
Vermetidae	*Dendropoma corrodens*	NP	0.612	Miloslavich and Penchaszadeh, 1992
	Vermetus sp.	NP	0.875	Miloslavich and Penchaszadeh, 1992
Siphonariidae	*Siphonaria serrata*	NP	0.731	Chambers and McQuaid, 1994
	Siphonaria concinna	P	0.996	Chambers and McQuaid, 1994
Trimusculidae	*Trimusculus conica*	NP	0.696	Haven, 1973

[1] P = planktotrophic; NP = nonplanktotrophic.
[2] Velar aspect ratio.

Hörstmann, 1956, for *Lymnaea stagnalis*). However, the presence of other potentially nutritive substances such as carbohydrates or lipids in the capsular fluid of *Littorina* has not been established.

Unlike the velum of species of *Littorina* with encapsulated, nonplanktonic development, the velum of planktotrophs does not appear to play a substantial role in the assimilation of intracapsular proteins. None of four plank-

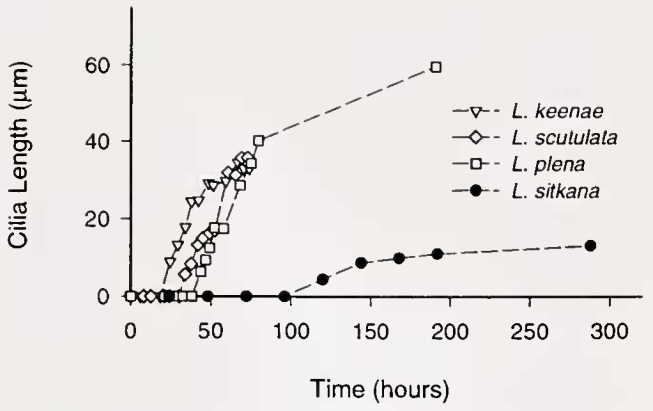

Figure 14. Lengths of the longest velar cilia over partial development of three planktotrophic (open symbols) *Littorina* and one *Littorina* with encapsulated, nonplanktonic development (filled circles). Cilia length units are micrometers and time units are hours, beginning at spawning of each species (time 0).

totrophic species exhibited localized affinity for FITC-BSA in the velum after larvae emerged from the egg envelope (pictured in Fig. 1). Preemergent larvae (still in the egg envelope) did not fluoresce after being soaked in FITC-BSA solutions, and one interpretation of these data is that larvae of planktotrophic species lack FITC-BSA affinity at this stage. A second possibility is that the egg envelope is not permeable to FITC-BSA, in which case preemergent larvae were never directly exposed to labeled protein. However, if the egg envelope is impermeable to large proteins, protein assimilation is certainly unlikely to be of nutritive importance to larvae contained in the very limited space within the egg envelope (see Fig. 1). Likewise, planktotrophs did not exhibit localized velar FITC-BSA affinity upon hatching from the egg envelope into the capsule space (when they would have access to any intracapsular protein) or after hatching from the egg capsule. Therefore, although at this point velar protein affinity cannot be conclusively ruled out for all stages of planktotrophic development, the velum of planktotrophs certainly does not function as a large assimilative surface for intracapsular proteins as it does in *Littorina* with encapsulated, nonplanktonic development.

Larvae of many planktotrophic marine invertebrate taxa have the ability to take up nutrients from seawater in the form of dissolved organic matter (DOM). Among molluscs, this ability has been demonstrated in bivalves (Manahan, 1983) and in the gastropod *Haliotis rufescens* (Jaeckle and Manahan, 1989). The primary site of DOM uptake in veliger larvae is the velum (Manahan and Crisp, 1983); however, because marine larvae take up DOM *via* carrier-mediated pathways (Wright and Manahan, 1989), this process is probably not homologous to velar protein uptake. Among gastropods, albumen is generally consumed by endocytosis (Elbers and Bluemink, 1960; Fioroni, 1977; Rivest, 1981; Rivest, 1992; Rivest and Strathmann, 1995).

Planktotrophy is the ancestral state in *Littorina*, so the presence of protein uptake by the larval epithelium of *Littorina* species with encapsulated, nonplanktonic development suggests that planktotrophic larvae may share a similar capability, although at a much lower level. The FITC-BSA assay used in this study and others (*e.g.*, Rivest, 1992; Rivest and Strathmann, 1995) may not be sufficiently sensitive to detect such low levels of protein endocytosis.

The evolutionary transition from planktotrophy to nonplanktotrophy is often accompanied by modifications to ancestral planktotrophic feeding characters (Strathmann, 1978). Such modifications have been viewed both as losses of complex structures that occur with the loss of planktotrophic larval feeding (Strathmann, 1978), and as novel features that enhance other aspects of nonfeeding larval performance (*e.g.*, swimming; Emlet, 1994). In gastropods, encapsulation of larvae is often associated with altered ciliary and velar morphology (Jägersten, 1972; Webber, 1977; Hadfield and Iaea, 1989). This study documents that compared to their planktotrophic congeners, veligers of *Littorina* with encapsulated, nonplanktonic development have shorter prototrochal cilia and cannot swim in seawater. Reductions in cilia length may be due to relaxation of selection for swimming and particle capture or, alternatively, to selection pressures associated with intracapsular development. Functions of prototrochal cilia in encapsulated larvae might include (1) rotating larvae to enhance oxygen diffusion through benthic, gelatinous egg masses (Hunter and Vogel, 1986; but see Strathmann and Strathmann, 1995), (2) feeding on nurse eggs (Lyons and Spight, 1973; Fioroni, 1988), or (3) stirring fluids to enhance feeding on intracapsular albumen. In the confined and more viscous environment of the egg capsule, short cilia may function better than long cilia in feeding on intracapsular nutrition or in rotating larvae.

The presence of recognizable planktotrophic larval feeding structures (such as the velum) in nonplanktotrophic species is commonly accepted as evidence of descent from a planktotrophic ancestor, and is one of many independent lines of evidence that support planktotrophy as the ancestral state in caenogastropods (Haszprunar *et al.*, 1995). Although various authors have remarked that encapsulated, nonplanktonic veligers have reduced velar lobes (*e.g.*, Jägersten, 1972; Webber, 1977; Rivest and Strathmann, 1995), possibly because the velum is no longer necessary for swimming and feeding (Jägersten, 1972), very few studies have addressed this issue in a comparative or quantitative context. The present study supports the general observation that during early development, gastropods with nonplanktotrophic development have smaller velar lobes than confamilial planktotrophs. Yet, degree of velar loss varies considerably: at one end of the range are species whose larvae never develop a velum (*e.g.*, Penchaszadeh and Rincón, 1996) and at the other are genera such as *Littorina* in which

planktotrophs and nonplanktotrophs have velums that are proportionally equivalent in early development. Retention of the velum may be attributed to several causes, including morphogenetic or other developmental constraints and an evolutionarily recent loss of planktotrophy. However, the lack of a velum in some species (*e.g.*, Penchaszadeh and Rincón, 1996) argues that the presence of velar lobes may not be necessary for prosobranch morphogenesis, and the second hypothesis has yet to be tested in a comparative, historical context. A third possibility is that nonplanktotrophs that retain a large velum do so in part because the velum has been coopted to perform specific functions in the egg capsule, such as feeding on nurse eggs (*e.g.*, Fioroni and Sandmeier, 1964) or providing a respiratory surface (Fretter and Graham, 1962). In *Littorina* with encapsulated, nonplanktonic development the velum performs a third and previously undescribed function, assimilation of intracapsular albumen. A large velum may be an advantage because it provides a large surface for assimilation of capsule proteins.

In summary, the velum of *Littorina* species with encapsulated, nonplanktonic development is active in endocytotic albumen assimilation. In contrast, affinity for labeled albumen was not seen in the velum of planktotrophic *Littorina* species. This suggests that, since their loss of planktotrophy, the former species have undergone evolutionary changes that enhance assimilation of intracapsular nutrients. The feeding morphologies of *Littorina* veligers differ in some ways, depending on developmental mode: species with encapsulated, nonplanktonic development have prototrochal cilia that are much shorter and grow more slowly, and the larvae cannot swim when removed from the egg capsule. However, the velar apparatuses of two *Littorina* species with contrasting development are comparable in size during early development. A survey of prosobranch gastropods suggests a general trend towards reduction in the relative size of the velum in nonplanktotrophic species. The size of the velum of *Littorina* with encapsulated, nonplanktonic development may be retained in part because of the velum's role in assimilation of intracapsular proteins.

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